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ABSTRACT

Islet-specific memory T cells arise early in type 1 diabetes (T1D), persist for long periods, perpetuate disease and are rapidly reactivated by islet transplantation. As memory T cells are poorly controlled by 'conventional' therapies, memory T-cell mediated attack is a substantial challenge in islet transplantation and this will extend to application of personalized approaches using stem-cell derived replacement β cells. New approaches are required to limit memory autoimmune attack of transplanted islets or replacement β cells. Here we show that transfer of bone marrow encoding cognate antigen directed to dendritic cells, under mild, immune-preserving conditions inactivates established memory $CD8^+$ T-cell populations and generates a long-lived, antigen-specific tolerogenic environment. Consequently, $CD8^+$ memory T cell-mediated targeting of islet-expressed antigens is prevented and islet graft rejection alleviated. The immunological mechanisms of protection are mediated through deletion and induction of unresponsiveness in targeted memory T-cell populations. The data demonstrate that hematopoietic stem cell-mediated gene therapy effectively terminates antigen-specific memory T-cell responses and this can alleviate destruction of antigen-expressing islets. This addresses a key challenge facing islet transplantation and importantly, the clinical application of personalized β-cell replacement therapies using patient-derived stem cells.

It is increasingly recognised that islet-specific memory T cells represent a major barrier to β-cell replacement therapies. Following immunogenic antigen encounter, naive T cells give rise to effectors and a small population of residual memory cells with increased longevity, faster response kinetics, and a reduced dependence on co-stimulatory signals (1). While this is advantageous for re-encounter with pathogens, formation of T-cell memory can be detrimental under some circumstances. Pathogenic memory T-cells are prominent in type 1 diabetes (T1D) where they arise during the early, preclinical phase of disease and are well established by the time of diagnosis (2). In addition to perpetuating disease, islet-specific $CD4^+$ and $CD8^+$ memory T cells are persistent and may remain dormant for extended periods after T1D onset but are rapidly reactivated by islet antigen exposure during islet transplantation and contribute substantially to rejection of transplanted islets (3,4). Thus, immunotherapy for T1D must address memory T cells regardless of disease stage, and this is crucial for success of islet transplantation.

Conventional immunosuppressants such as calcineurin inhibitors and rapamycin control memory much less effectively than naive T-cell responses (5). Many immunotherapies for T-cell mediated diseases attempt to reintroduce or expand regulatory T cells (Treg), however, memory T cells can resist suppression by Treg (6,7). Therefore, development of new approaches to terminate memory T-cell responses in a controlled, antigen-specific context would be highly beneficial. In the clinical context, this is particularly pertinent for application of islet transplantation and personalized β-cell replacement therapies using patient-derived stem cells where memory autoimmune responses would attack replacement cells.

Dendritic cells are increasingly considered clinically-appropriate therapeutics for T1D and other autoimmune diseases. Indeed, memory $CD4^+$ and $CD8^+$ T-cell responses are terminated by

dendritic cells (DC) or other antigen-presenting cells (APC) engineered to express cognate antigen (8-10). One strategy to achieve APC-targeted antigen expression to prevent diabetes development therapeutically is to transfer bone marrow (BM) or hematopoietic stem and progenitor cells (HSPC) genetically-modified to encode antigen expression that, once engrafted, continually gives rise to antigen-expressing APC (11). Autologous hematopoietic stem cell transplantation (HSCT) has been trialed for T1D with exciting outcomes but, as currently applied, uses toxic conditioning leading to complete immune ablation to deplete pathogenic immune cells including memory T cells (12,13).

Here we tested the hypothesis that addition of gene therapy to HSCT, to achieve tolerogenic self-antigen expression in APC, would terminate established memory T-cell responses, restoring long-lasting tolerance to islet antigens whilst maintaining protective immunity. We demonstrate that, under immune-preserving conditions, transplantation of BM encoding DC-expressed antigen terminates cognate $CD8^+$ memory T cell responses and alleviates immune destruction of newly-transplanted islets.

RESEARCH DESIGN & METHODS

Mice

OT-I (14), 11c.OVA (15) and K5.mOVA (16) mice were maintained under SPF conditions in the PAH BRF or TRI BRF, Brisbane, Australia. C57BL/6 and B6.SJL*ptprca* mice were purchased from ARC (Perth, Australia). B6.SJL and OT-I mice were crossed to generate $CD45.1^+CD45.2^+$ OT-I mice. 11c.OVA mice were backcrossed to B6.SJL*ptprca* to make $CD45.1^+$ 11c.OVA mice. Unless stated otherwise, $CD45.1^+$ B6.SJLptprca mice were used as recipients and $CD45.2^+$ mice used as BM donors. All animal procedures were approved by the University of Queensland Animal Ethics Committee.

OT-I T-cell transfers

To transfer naïve OT-I T cells, suspensions of pooled mesenteric, inguinal, axillary, and brachial lymph nodes (LN) were prepared and transferred ($5x10^6$) as described (15). Memory OT-I T cells were generated as described previously (8). Briefly, lymph nodes were harvested from OT-I or $CD45.1^+CD45.2^+$ OT-I mice and cultured in complete RPMI with 1% mouse serum, 0.1 µg/mL $OVA_{257-264}$ and 10ng/ml rhIL-2. After 3 days, cells were washed and recultured with 10 ng/mL rmIL-15 for 2 days. Where indicated, OT-I T cells were CFSE labelled before transfer. Unless stated otherwise, $2x10^6$ Tmem were transferred (i.v. lateral tail vein).

Bone marrow transplantation

Femurs and tibias were collected into mouse-tonicity (MT)-PBS. BM was flushed with MT-PBS/2.5% FCS, erythrocytes lysed (NH_4Cl/TRIS buffer) and BM injected i.v. within 3 hours of irradiation (300cGy, ^{137}Cs source). HSPC for transfer were prepared by high speed FACS

sorting of lin^{-ve}c-kit^{+ve} cells to typically >95% purity from bulk BM. HSPC were depleted from BM by sorting lin^{+ve}c-kit^{-ve} cells from BM.

In vitro and in vivo assays

CFSE labelling and flow cytometric analyses including bead-based counting assays were performed as previously described (15,17). OVA/QuilA immunisation was as described (9). In vivo CTL assays were preformed as previously described (15). mAb were purchased from Biolegend, BD Biosciences, BioXcell (Lebanon, NH) or grown, purified, and conjugated in-house. ELISpot assays were as described (15) using $OVA_{257-264}$ (0.5μg/mL) or KLH (10μg/mL) stimulation and counted with an ELISpot reader (AID GmbH, Strassburg, Germany).

Skin and islet transplantation

Skin grafting used double grafting of skin from K5.mOVA and BALB/c donors as described (18). For islet transplantation, mice were administered a single dose of streptozotocin (STZ, 200 mg/kg, pH 4.2 citrate buffer) i.p. and blood glucose (BG) monitored daily using an Accu-Check Advantage (Roche). Hyperglycemic (>16.6 mM) mice were transplanted under the kidney capsule with islets prepared from OVA^{+ve} RIP.mOVA or OVA^{-ve} littermate donors at a ratio of 3 donors per recipient. Blood glucose was monitored as indicated. Mice in which euglycemia (<16.6mM) was not initially restored were excluded due to primary graft non-function. Rejection was defined as 2 consecutive BG >16.6 mM after initial euglycemia. Mice were nephrectomised ≥100 days post-grafting to determine graft-dependence of euglycemia. Kidneys containing islet grafts were formalin fixed and embedded for histological analysis (19).

Statistical analysis

Student's *t*-test was used for comparison of means and one-way ANOVA with Newman-Keuls or Tukey's post-test for multiple comparisons (GraphPad Prism 5 or Prism 6). Survival data was analysed using a Log-rank test (Mantel-Cox) (GraphPad Prism 5 or Prism 6).

RESULTS

In vitro generated OT-I Tmem establish functional in vivo memory populations

To study induction of tolerance to islet-antigen, we adapted an established system (8,20) to generate and monitor $CD8^+$ T-cell memory at the individual cell level. Lymph node cells from OVA-specific OT-I TCR transgenic mice were cultured in peptide/IL-2 washed and cultured in IL-15 over a period of 5 days (8,21) which gives rise to predominantly $CD44^{hi}/CD62L^+/CD69^-$ central memory phenotype cells referred to here as Tmem. These cells establish long-lived memory populations in vivo that readily respond to immunogenic rechallenge **(Fig. 1A)** which would target OVA-expressing β cells.

Low-dose irradiation permits effective engraftment of antigen-encoding BM whilst preserving $CD8^+$ T-cell memory

Low-dose irradiation facilitates engraftment of BM in naive and primed mice (22) and preserves immunity. To test whether immunity was preserved in mice with TCR-transgenic memory $CD8^+$ T-cells, OT-I Tmem were transferred to $CD45.1^+$ recipients. Recipients were left untreated or irradiated 1 week later (300cGy) and BM from $CD45.2^+$ non-transgenic, mice transferred. No significant loss of responsiveness in OT-I memory populations resulted from low-dose irradiation and transfer of non-Tg BM **(Fig. 1B)**. Antigen-encoding BM engraftment in immune-competent mice is enabled by restricting antigen expression to differentiated $CD11c^+$ DC (22). To test this in mice carrying TCR transgenic memory $CD8^+$ T-cells, OT-I Tmem were transferred and recipients irradiated (300cGy) 1 week after transfer and then BM from $CD45.2^+$ non-transgenic or 11c.OVA donors, which express OVA in DC (15), transferred. Donor-type ($CD45.2^+$) leukocytes accumulated in blood and spleen, indicating BM effectively engrafted from OVA-negative and 11c.OVA donors **(Fig. 1C,D)**. Donor-type DC developed similarly in

recipients of 11c.OVA or non-Tg BM **(Fig. 1E)** indicating OVA expression was no impediment to DC development in the presence of OVA-specific Tmem.

Transplantation of antigen-encoding bone marrow inactivates OT-I memory T cells

We next determined whether antigen-encoding BM transfer under immune-preserving conditions inactivated pre-existing memory $CD8^+$ T-cell responses. OT-I Tmem were transferred to non-Tg mice and 1 week later, mice were irradiated (300cGy) and 11c.OVA or non-Tg BM transferred. Six weeks later, responsiveness of OT-I cells was tested by immunogenic challenge with OVA/QuilA (8) which substantially expanded OT-I T-cell number in spleens of no-BMT controls and non-Tg BM recipients relative to sham-challenged controls (**Fig. 2A-C**) but not in recipients of 11c.OVA BM (**Fig. 2A-C**). Intracellular cytokine staining for IFN-γ, as a measure of effector function, showed OVA/QuilA challenge elicited an approximately 200-fold increase in total IFN-γ-producing OT-I T cells in spleens of both no BMT controls and non-Tg BM recipients (**Fig. 2D**). The number of splenic IFN-γ-producing OT-I T-cells in 11c.OVA BM recipients remained low, however, regardless of challenge **(Fig. 2D)**. Similarly, little CTL activity was elicited *in vivo* by OVA/QuilA challenge of 11c.OVA BM recipients, but CTL activity was substantial in recipients of non-Tg BM or in no BMT controls **(Fig. 2E)**. As we have reported previously (22), BMT using low-dose irradiation does lead to some mild, mostly non-significant reduction in responsiveness to OVA-challenge (Fig. 2A-D). Together this indicates responsiveness of an established OT-I Tmem population was effectively inhibited by OVA-encoding BM transfer. In additional studies where OT-I Tmem recipients were immunised with KLH/QuilA prior to BM transfer, KLH responses were preserved regardless of whether OVA-encoding or non-Tg BM was transferred (**Supplementary Fig. 1**) indicating antigen-specificity of the tolerogenic effect of OVA-encoding BM transfer.

We observed no change in the proportion of donor-type DC in either non-Tg or 11c.OVA BM recipients after OVA/QuilA challenge (**Fig. 2F**) indicating the lack of $OVA_{257-264}$-specific CTL activity in 11c.OVA BM recipients (**Fig. 2E**) is attributable to inactivation rather than redirection of OVA-specific CTL activity to cytotoxic destruction of OVA-expressing DC.

Bone marrow transfer is effective at high CD8$^+$ memory T cell frequencies.

To determine whether the effectiveness of Ag-encoding BM transfer was limited if Ag-specific Tmem were highly abundant, a large number ($4x10^7$) of OT-I Tmem were transferred and then non-Tg or 11c.OVA BM transferred. OVA/QuilA challenge 4 weeks after BM transfer, showed that while OT-I Tmem retained responsiveness and the capacity to proliferate and produce IFN-γ in recipients of non-Tg BM (**Fig. 2G,H)** this was completely ablated in recipients of OVA-encoding 11c.OVA BM **(Fig. 2G,H).** This indicates that Ag-encoding BM transfer is an effective strategy to achieve antigen-specific tolerance, particularly for Tmem, even when the frequency of Ag-specific CD8$^+$ Tmem is very high, as might occur in T1D.

Transplantation of antigen-encoding bone marrow prevents islet rejection

We sought to determine whether ablation of a cognate pre-existing memory CD8$^+$ T-cell response might prevent memory T-cell mediated rejection of 'replacement' tissues. For islet transplants this would model the autoimmune component of responses mounted against islet grafts in T1D where, here, OVA substitutes for β-cell autoantigens. Memory CD8$^+$ T-cell were established by Tmem transfer and one week later mice were left untreated or irradiated (300cGy) and non-Tg or 11c.OVA BM transferred. Four weeks later, mice were rendered diabetic by STZ treatment and islets from syngeneic OVA^{+ve} RIP.mOVA mice expressing OVA in pancreatic β cells or OVA^{-ve} littermate controls were transplanted under the kidney capsule and glycemia monitored to determine islet graft survival. OVA^{-ve} islets transplanted as technical controls

survived long-term, stably restoring euglycemia in a graft-dependent manner (**Fig. 3A**, **Supplementary Fig. 2**). When grafted into OT-I Tmem recipients to which control non-Tg BM had been transferred, 5 of 8 OVA-expressing islet grafts failed to stably restore euglycemia and graft failure occurred soon after transplantation in most cases (**Fig. 3A, Supplementary Fig. 2**). In contrast, in mice carrying memory OT-I T cells that received OVA-encoding 11c.OVA BM, 8 of 9 islet grafts stably restored euglycemia (**Fig. 3A, Supplementary Fig. 2**). Insulin-positive cells were prominent in islet grafts of mice that received 11c.OVA BM (**Fig. 3B**).

To further define the ability of antigen-encoding BM transfer to prevent rejection of transplanted antigen-expressing tissues we compared survival of allogeneic and OVA-expressing skin grafts. OT-I Tmem were transferred to non-Tg recipients and non-Tg or 11c.OVA BM transferred after low-dose irradiation. Six weeks later, recipients and no-BMT controls were double-grafted with skin from allogeneic BALB/c and syngeneic K5.mOVA donors expressing OVA in skin. Control, no BM transfer Tmem recipients rejected >80% of OVA-expressing skin grafts rapidly, as did recipients of non-Tg BM. Remaining grafts promptly rejected in response to OVA/QuilA challenge 100 days after placement (**Fig. 3C**) indicating residual OVA-specific memory. Profoundly, almost two thirds of OVA-expressing skin grafts survived long-term in the 11c.OVA BMT group (**Fig. 3C**) and none rejected in response to OVA QuilA challenge indicating robust graft acceptance in this group. In all mice tested, allogeneic skin rejected with a similar tempo (**Fig. 3D**) indicating no gross immunosuppression that might contribute to a difference between test groups. These data demonstrate successful induction of tolerance which alleviates Tmem attack of antigen-expressing islets and skin, addressing a key challenge facing islet transplantation and personalized β-cell replacement therapies.

Tmem inactivation requires engraftment of antigen-encoding hematopoietic progenitors

As gene-engineered BM transfer could have clinical application we sought to understand the mechanisms of tolerance induction. To define whether engraftment of hematopoietic stem and progenitor cells (HSPC) was required and to negate any contribution from differentiated cell co-transferred in BM such as Treg, sort-purified $lin^{-ve}c\text{-}kit^{+ve}$ HSPC were transferred to mice carrying OT-I Tmem under engrafting (300cGy) or non-engrafting (no irradiation) conditions and tolerance induction tested by OVA/QuilA challenge. Donor-type leukocyte accumulation, indicating engraftment, was lower in HSPC than BM recipients (**Fig. 4A**) as anticipated from previous studies (22). However, 5 weeks after HSPC or BM transfer, total spleen cell number did not differ between groups (**Fig. 4B**). Whether mice received 11c.OVA HSPC or BM, OVA/QuilA elicited little expansion of the memory OT-I population as expected (**Fig. 4C**). Substantial OT-I expansion, similar to that in non-Tg BM or HSPC recipient controls occurred, however, if mice received HSPC-depleted 11c.OVA BM under engrafting conditions or 11c.OVA HSPC under non-engrafting conditions. Together this indicates engraftment of antigen-encoding HSPC is necessary and sufficient for antigen-specific $CD8^+$ tolerance and non-engrafting components of BM do not contribute. A prerequisite for tolerance induction is steady-state antigen presentation by antigen-expressing DC (15). Transfer of CFSE-labelled OT-I T cells revealed 11c.OVA BM transfer, led to long-term presentation of OVA determinants for at least 12 weeks in recipient mice **(Fig. 4D)**. Furthermore, in addition to ablating responsiveness of antigen-specific memory $CD8^+$ T cells, OVA-encoding BM establishes a long-term tolerogenic environment in recipients (**Supplementary Fig. 3**) that would prevent re-emergence of targeted antigen-specific T-cell populations.

Ablation of memory OVA-specific $CD8^+$ T-cell responses by OVA-encoding BM does not establish OVA-specific 'regulation'

Using irradiation to facilitate BM engraftment has been associated with generation or expansion of $CD4^+$ or $CD8^+$ regulatory T-cell populations that modulate subsequent immune responses (23,24). To determine whether OVA-encoding BM transfer induced a regulatory response that inhibited subsequent OVA-specific T-cell activation we adapted a widely used 'regulation' assay and compared proliferation of a second adoptively-transferred population of OVA-specific $CD8^+$ T cells in mice where OVA-specific memory $CD8^+$ T-cells had been inactivated or not by BM transfer. Mice were injected with $CD45.2^+$ OT-I Tmem or not and one week later irradiated (300cGy) and non-Tg or 11c.OVA BM transferred. Six weeks later, a new cohort of congenically-distinct ($CD45.1^+$) CFSE-labelled naive OT-I cells were transferred as the 'test' population and CFSE dilution measured 3 days later. To provide a consistent source of antigen stimulation between recipients of non-Tg and OVA-encoding BM, some mice were immunised subcutaneously with OVA/QuilA. In non-Tg BM recipients, CFSE dilution indicated OT-I T cells divided only after immunisation (**Fig. 5A**, left and centre panels) and proliferation was most extensive in LN draining the site of immunisation (**Fig. 5A**, centre panels). In non-draining LN (non-dLN) where immunisation-derived OVA is not presented, proliferation was most extensive in 11c.OVA BM recipients (**Fig. 5A**, centre and right lower panels). However, after immunisation, proliferation of test T cells was similar in draining LN (dLN) of non-Tg and 11c.OVA BM recipients (**Fig. 5A**, centre and right upper panels) and this was not altered by the presence of the initially-transferred, but tolerised OT-I Tmem (**Fig. 5B,C**). Together. these observations show that 11c.OVA BM transfer, whether or not in the presence of OT-I Tmem, had not established OVA-specific regulation. In line with this, no significant differences were observed in the proportion of $CD4^+CD25^+FoxP3^+$ Treg between groups and transfer of 11c.OVA did not lead to differentiation of OT-I Tmem to $CD8^+FoxP3^+$ Treg (**Fig. 5D**).

Deletion contributes to ablation of CD8$^+$ memory responses after transfer of antigen-encoding BM

Serial analyses of peripheral blood (e.g. **Fig. 1A**) indicated that after an initial expansion phase, the population of OT-I T cells may have been contracting, possibly through deletion. When we serially-sampled blood over time, the frequency of OT-I increased similarly in non-Tg and 11c.OVA BM recipients for 3-4 weeks, but then appeared to contract in 11c.OVA BM recipients (**Fig. 6A**). In spleens, where only late time-points were analysed, a similar pattern was apparent and the number of OT-I T cells significantly dropped in 11c.OVA BM recipients between 28 and 49 days after BM transfer (**Fig. 6B**). Together, this was consistent with a pattern of OT-I Tmem expansion followed by deletion in 11c.OVA BM recipients that would be anticipated based on previous studies (22). As host T-cell dynamics after low-dose irradiation might confound this analysis we tested the contribution of deletion more directly. The proapoptotic bcl-2 family member bim is a crucial mediator of DC-induced T-cell deletion (25). Therefore, we transferred deletion-resistant OT-I.bim$^{-/-}$ Tmem or deletion-competent OT-I Tmem and 1 week later BM from non-Tg or 11c.OVA mice was also transferred. OT-I and OT-I.bim$^{-/-}$ T cells were enumerated in peripheral blood 1 day before and at regular intervals after BM transfer. In recipients of non-Tg BM, few OT-I T cells accumulated in blood and the number of these diminished slowly with time (**Fig. 6C**). In 11c.OVA BM recipients, WT OT-I T cells expanded and then began to diminish in number consistent with slow contraction of the population. On the other hand, deletion-impaired bim-deficient OT-I T cells continued to expand and accumulated in many-fold greater numbers than WT equivalents in blood (**Fig. 6C**) and in spleen (**Fig. 6D**). Together this demonstrates bim-mediated deletion limits OT-I T accumulation after transfer of 11c.OVA BM and enforces contraction of the OVA-specific Tmem population.

Residual, unresponsive CD8$^+$ memory cells exhibit a CD5^{hi} phenotype distinct from 'exhaustion'

Although OT-I Tmem undergo deletion after 11c.OVA BM transfer, the number of residual OT-I Tmem in spleen is similar to or higher than in recipients of non-Tg BM (e.g **Fig. 1C, Fig. 2C**) but functional studies (**Fig. 1, Fig. 2**) indicate these cells are inactivated. Long-term expression of OVA by DC in 11c.OVA BM recipients (**Fig. 5**) could induce inactivation by 'exhaustion' or other mechanisms. To investigate this, OT-I Tmem were transferred to recipient mice and then 11c.OVA or non-Tg BM transferred and residual Tmem phenotyped 4 weeks later. OT-I Tmem were also transferred to unirradiated 11c.OVA or non-Tg controls (Fig.7, no BMT) for parallel analysis. 'Exhaustion' is a prominent mechanism inhibiting CD8$^+$ T-cell effector function, particularly in chronic viral infection and cancer where 'inflammation' or upregulation of co-inhibitory ligands may be present (26). As PD-1 is considered an archetypal marker of exhaustion and has been implicated in induction and maintenance of tolerance we examined expression of PD-1 and a range of other markers associated with 'exhaustion'. In non-Tg BM recipients and non-Tg controls, PD-1 was expressed at low levels by around one-third of residual OT-I cells (**Fig. 7A-C**). In 11c.OVA BM recipients, PD-1 was expressed by the majority of cells at moderate levels (**Fig. 7A-C**). In 11c.OVA controls, PD-1 was expressed on almost all residual OT-I Tmem and at high levels relative to the other experimental groups. CD5 negatively regulates intracellular signalling in lymphocytes and dampens T-cell responsiveness by inhibiting TCR signalling (27). CD5 expression has, however, not typically been associated with chronic viral infection-induced exhaustion but rather with T-cell 'tuning' in response to chronic antigen exposure. Relatively little CD5 was expressed by OT-I in non-Tg BM recipients and non-Tg controls but a small population expressed moderate levels (**Fig. 7A,B**). Almost all

residual OT-I in 11c.OVA BM recipients and 11c.OVA controls expressed high levels of CD5, mostly co-expressed with PD-1, particularly in 11c.OVA controls (**Fig. 7A**) which reflected overall expression levels of CD5 and PD-1 (**Fig. 7B,C**). Consistent with expression of OVA by all DC in the 11c.OVA control OT-I recipients, compared to the approximately 30-40% in 11c.OVA BMT mice, the overall expression of both PD-1 and CD5 was significantly higher on OT-I T cells from 11c.OVA control recipients than those from mice that received 11c.OVA BM transfers (**Fig. 7B,C**). Of a range of surface markers indicative of T-cell exhaustion, CD160 and to a lesser extent CD244 were expressed by residual OT-I T cells only in non-Tg BM recipients and non-Tg controls whereas TIM-3 and LAG-3 were not expressed.

DISCUSSION

The autoimmune anti-β cell response reawakened by islet transplantation is a significant component of recipient immune resistance to islet transplants and remains a significant clinical challenge. Because memory T cells are difficult to control with conventional immunosuppression (3) and are resistant to, for example, cytotoxic drugs and the inhibitory effects of regulatory T cells (6,7,17,28), unwanted, detrimental memory T-cell responses are difficult to control using conventional therapies (5). Thus, current therapeutic approaches are poorly effective or have substantial detrimental side-effects and new approaches are required to control pathogenic memory T-cell responses. Here, using transfer of BM or HSPC genetically-encoding cognate antigen expression under mild, immune-preserving pre-BMT conditioning, we demonstrate a highly-effective alternative to current therapies. This approach, which antigen-specifically ablates pathogenic memory T-cell responses, provides an avenue by which autologous HSC transplant (aHSCT) therapies could be modified and optimised to avoid immune suppression whilst antigen-specifically targeting and preventing the re-emergence of detrimental memory T-cell responses.

Memory T cells represent the immunological driving-force in many pathological states. As they are programmed to rapidly expand and generate large effector populations upon antigen re-encounter they pose a significant barrier to tissue, cell and protein replacement therapies in 'primed' individuals. Due to their terminal differentiation and costimulation-independence it has long been considered that memory T cells are resistant to tolerance induction (29,30). However, pioneering studies including our own where antigen expression has been enforced in APC, have shown that memory $CD4^+$ or $CD8^+$ T-cell responses can be silenced (8-10,31-33). We now advance this to show application to the clinical challenge of β cell-specific memory T-cell

responses that represent a strong impediment to islet graft survival. Previous studies showed when using gene-modified BM transfer that restricting antigen expression to differentiated APC permitted BM engraftment under immune-preserving conditions in naive and primed recipients (22). However, the fate of established memory T cells was not examined. Mechanistic studies here show that using this approach, memory $CD8^+$ T-cell populations are inactivated through a combination of deletion and induction of unresponsiveness in residual undeleted cells. Here we identified deletion by using T-cell donors with a complete functional loss of bim. Altered function of some of the bcl family of apoptosis regulators and in deletional pathways has been reported in the NOD mouse (34) and may exist in humans with T1D. While potentially contributing to disease, these alterations are subtle and not comparable to complete gene knockout. In NOD mice, these alterations are unlikely to have a significant impact on tolerance as our previous studies (11) indicate that enforced expression of the proinsulin islet antigen is sufficient to promote tolerance in this strain. Overall, the data indicate that residual OT-I remaining after deletion which are rendered unresponsive do not exhibit a classical 'exhausted' phenotype, but a phenotype that suggests modulation of responsiveness by mechanisms such as 'tuning' - as indicated by PD-1 and CD5 expression - of TCR responsiveness. In the BM transfer setting, approximately 30-40% of DC express OVA and, accordingly, OT-I expression of PD-1 and CD5 is reduced compared to OT-I T cells transferred to 11c.OVA mice where all DC express OVA. This is consistent with previous reports that T-cell 'tuning' is dependent on antigen expression levels (35,36). The data also indicate that inactivation is effective even when antigen is expressd by a minority of all DC. The current study specifically examines the response of $CD8^+$ T cells. We have reported previously that the presence of cognate $CD4^+$ T cells alters the kinetics, but does not prevent tolerance induction (15) and that adoptively-

transferred memory $CD4^+$ T cells are inactivated (10) when antigen is targeted to DC. In studies where $CD4^+$ T cells have been adoptively transferred to recipients of gene-engineered BM, differentiation of $CD4^+$ Treg has been reported (37). Such induced $CD4^+$ Treg are likely to provide bystander suppression and provide additional therapeutic value. Therefore, we propose this approach would also be effective for control of pathogenic memory CD4+ T-cell responses.

One current approach used clinically to ablate pathogenic T-cell responses in severe autoimmune disease is aHSCT. This approach, as currently practiced, while largely effective, relies on the use of cytotoxic agents, typically with or without T-cell depletion (38), to ablate the entire immune repertoire and achieve an immune 'reset' in the absence of specific mechanisms leading to protective, antigen-specific tolerance. Some studies have tested the principle of antigen-encoding BM transfer in a 'primed' setting using 'non-myeloablative' approaches, but these have exclusively employed T-cell depletion or other immunodepleting strategies (39-41) so that while recipients are ultimately reconstituted with a 'tolerant' T-cell repertoire, this is not 'immune-preserving'. Our findings represent a significant step forward by providing an immune-preserving procedure and predicate a potential clinically-applicable approach to ablating unwanted memory T-cell responses. Additionally, this demonstrates induction of mixed allogenic chimerism, with its attendant risks, is not required. For instance, the risk of graft-versus-host disease is negated as the donor cells are syngeneic or autologous to recipients. Similarly, for islet transplantation in T1D there is no risk of disease exacerbation as replacement islets would be transplanted after T-cell inactivation is complete. If this procedure were to be used to control autoaggressive T-cell responses in at-risk individuals, then transient inflammatory exacerbation could potentially be controlled by a short course of immunosuppression.

Allogeneic islet or whole pancreas transplantation is currently the only 'curative' therapy for T1D but its application is limited by several factors including insufficient donor pancreata and the substantial side-effects of long-term immunosuppression. With significant advances made recently in generation of stem cell-derived β cells (42,43) and genetically-engineered insulin-secreting cells (44,45) it is conceivable that generation of personalised insulin-secreting cells will be feasible in the future. However, persistent anti-islet memory T-cell responses represent a threat to clinical application of such approaches. Further development of gene-engineered BM transfer protocols may lead to regimens that can be applied with less risk than current organ transplant procedures. Under these circumstances, employing gene-engineered BM to ablate memory T cell responses could reduce the requirements for immunosuppression in allogeneic islet transplantation or in the case of personalised autologous replacement avoid the need for immunosuppression completely.

The present study was designed to provide proof-of-principle pre-clinical data and to define the mechanistic contributions to the therapeutic effects of BMT on autoaggressive memory $CD8^+$ T cells. Nevertheless before this approach can be translated, a number of challenges remain. An ideal translational application of the single antigen approach demonstrated in these pre-clinical studies would be to protect insulin-secreting cells engineered from non-beta cells such as hepatocytes (44,45). However, prevention of T1D progression in at-risk or recent-onset human subjects may require a more complex approach. In an appropriate pre-clinical model, it would be important to demonstrate simultaneous inactivation of responses to multiple relevant antigens, and/or bystander tolerance where regulatory T cells induced towards one antigen regulate immunity towards additional relevant local antigens. Additionally, interpretation based on TCR transgenic T cells may be limited and in future studies endogenous antigen-specific cells would

need to be examined using tetramers. Humanised mice grafted with relevant human tissue antigens may represent one pre-clinical model where such advances could be made. Finally, mild conditioning regimens would need to be developed to support HPSC transfer in young individuals with, or at risk of, T1D. Notwithstanding these challenges we demonstrate a highly effective method to silence autoaggressive islet-destructive memory T cell responses that, with further development, has clinical potential.

AUTHOR CONTRIBUTIONS

MAC, CFJ, RT, STG, PTC, JMF, JWW, RJS designed experiments. MAC, CFJ, DP, SW, DJB, RG, JAB, NHO performed experiments. MAC, CFJ, SW, RJS analysed experiments. MAC, STG, RT, RJS wrote the manuscript.

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CONFLICT OF INTEREST DISCLOSURES

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Immune-preserving conditioning permits engraftment of 11c.OVA bone marrow in the presence of OVA-specific memory CD8$^+$ T cells. **A)** OT-I Tmem ($2x10^6$) were transferred to non-Tg (C57BL/6, CD45.2$^+$) mice that were sham (PBS/QuilA) or OVA/QuilA challenged 6 weeks later. One week later OT-I (CD45.1$^+$CD45.2$^+$CD8$^+$Vα2$^+$) T cells were enumerated. Fold expansion is shown in brackets. **B)** OT-I Tmem were transferred to non-Tg mice and 1 week later recipients left untreated or irradiated (300cGy) and injected i.v. with non-Tg BM (10^7). 6 weeks later mice were sham (PBS/QuilA) or OVA/QuilA challenged. OT-I T-cells were enumerated 1 week later. Fold expansion is shown in brackets. **C-E)** OT-I Tmem were transferred to non-Tg mice and 1 week later recipients irradiated (300cGy) and non-Tg or 11c.OVA BM (10^7) injected i.v. Peripheral blood as indicated (**C**) or spleen at 7 weeks (pooled sham-challenged & challenged mice) was analysed (**D**) and donor-type DC (CD11c^{hi}) were enumerated in peripheral blood (**E**).

Figure 2. Inactivation of memory OT-I T-cell populations by OVA-encoding BMT. A-F) OT-I Tmem (CD45.1$^+$/CD45.2$^+$) were transferred to non-transgenic mice (CD45.1$^+$) and 1 week later mice were irradiated (300cGy) or not and non-Tg or 11c.OVA BM (CD45.2+, 10^7) transferred. 6 weeks after BMT mice were sham- or OVA-challenged and 1 week later or, as indicated, analysed by flow cytometry. OT-I T cells CD45.1$^+$/CD45.2$^+$CD8$^+$Vα2$^+$) were serially monitored in blood by cytometry (**A**) or 1 week after challenge (**B**). Spleens were analysed 1 week after OVA/QuilA challenge (**C,D,F**). In vivo CTL was performed 1 week after challenge (**E**). **G,H)** OT-I Tmem ($4x10^7$, CD45.1$^+$/CD45.2$^+$) were transferred to non-transgenic mice (CD45.1$^+$) and 1 week later mice were irradiated (300cGy) and non-Tg or 11c.OVA BM

(CD45.2+, 10^7) transferred. 4 weeks after BMT mice were sham- or OVA/QuilA-challenged and 1 week later analysed by flow cytometry. Total number of OT-I per spleen (**G**) and total IFN-γ+ OT-I per spleen (**H**) was determined. Data are pooled from 3 experiments and represent mean ± SEM (n=6-11) (**A**) or individual mice with mean ± SEM (**B-F**) or pooled from 2 separate experiments showing individual mice with mean ± SEM (**G,H**)

Figure 3. Antigen-encoding BM transfer promotes survival of antigen-expressing islet and skin grafts. A,B) OT-I Tmem ($4x10^7$) were transferred to non-transgenic mice. One week later mice were irradiated (300cGy) and non-Tg or 11c.OVA BM transferred. Four weeks after BM transfer, mice were rendered diabetic with streptozotocin (200mg/kg) and islets from RIP.mOVA mice transplanted under the kidney capsule. Blood glucose was monitored and rejection determined as 2 consecutive readings >16.6mM. Graft sites were embedded, sectioned and stained for insulin (**B**). **C,D)** OT-I Tmem were transferred to non-transgenic mice. One week later mice were irradiated (300cGy) and non-Tg or 11c.OVA BM transferred. Six weeks after BMT, mice were double grafted with BALB/c and K5.mOVA skin. Mice retaining skin grafts at 100 days post-grafting were immunised with OVA/QuilA. Data are pooled from 2-3 experiments per group.

Figure 4. Termination of $CD8^+$ Tmem responses requires HSPC engraftment. A-C) OT-I Tmem were transferred to non-Tg mice that were irradiated (300cGy) or not 1 week later and BM, lin^{-ve}/$c\text{-}kit^{+ve}$ HPC or BM depleted of lin^{-ve}/$c\text{-}kit^{+ve}$ HSPC transferred as indicated. 4 weeks after BM transfer mice were challenged or not with OVA/QuilA and 1 week later analysed. Donor engraftment (**A**), total spleen cell number (**B**) and OT-I in spleen (**C**) were determined by

flow cytometry. **D)** Mice were irradiated (300cGy) and non-Tg or 11c.OVA BM (10^7) transferred. CFSE-labelled naive OT-I T cells were transferred as indicated and analysed 3 days later. Proliferation index is indicated (mean ± SD, n=4). Data are pooled from 4 experiments (**A-C**) and show individual mice (mean ± SEM, n=4 (sham) or 8 (immun.) per group) or representative of 4 mice from 2 separate experiments.

Figure 5. Transfer of OVA-encoding BM does not establish OVA-specific regulation. A-C) OT-I Tmem were or were not transferred to non-Tg recipients. One week later mice were irradiated (300cGy) and non-Tg (C57BL/6) or 11c.OVA BM transferred. Six weeks after BM transfer, $5x10^6$ naive CFSE-labelled OT-I cells were transferred and mice immunised with OVA/QuilA subcutaneously. CFSE dilution in the draining (inguinal) and non-draining (pooled axillary, mesenteric) lymph nodes was assessed 3 days later by flow cytometry. **D)** OT-I Tmem were transferred to non-Tg mice that were irradiated (300cGy) and injected i.v. with BM from non-Tg or 11c.OVA donors (BMT) 1 week later. Controls (no BMT) were non-Tg or 11c.OVA mice injected with Tm in parallel with BM transfer test mice. Four weeks after BM transfer, spleen cells were examined by flow cytometry. Data are representative of 6 mice in 2 separate experiments (A) or pooled from two separate experiments (B-D). Lines depict mean ± SEM (n=4-6 per group).

Figure 6. Deletion is an important contributor to ablation of pre-existing memory $CD8^+$ T-cell responses. A,B) OT-I Tmem ($CD45.1^+/CD45.2^+$) were transferred to non-Tg mice (B6.SJL, $CD45.1^+$) and 1 week later mice were irradiated (300cGy) and injected i.v. with BM (10^7) from non-Tg (C57BL/6, $CD45.2^+$) or 11c.OVA ($CD45.2^+$) donors. At the indicated time

points, blood or spleen was examined by flow cytometry. **C,D)** WT or bim$^{-/-}$ OT-I Tmem (CD45.2^{+}, 2x10^{6}) were transferred to non-Tg (B6.SJL, CD45.1^{+}) mice. One week later mice were irradiated (300cGy) and injected i.v. with BM (10^{7}) from non-Tg (B6.SJL, CD45.1^{+}) or 11c.OVA (CD45.1^{+}) donors. OT-I T cells were monitored in blood the day before BM transfer or at the indicated points (**C**) or in spleen at the end of the experiment (38 days post-BMT) (**D**). Data are pooled from 2-3 experiments per time point with typically 2-4 mice per group in some of which mice were sham-immunised (PBS/QuilA) at day 42 (**A,B**) or pooled from 2 experiments (**C,D**). Data show individual points or mean ± SEM. B) * 11c.OVA BM 49 days after BM transfer is significantly lower than 11c.OVA BM 28 days after BM transfer ($p<0.05$) **C**) ** OT-I.bim$^{-/-}$ 11c.OVA BM is significantly greater than WT OT-I 11c.OVA BM transfer at days 23, 37 ($p<0.001$), ** OT-I.bim$^{-/-}$ 11c.OVA BM days 23, 37 significantly greater than days 11, 18 ($p<0.001$).

Figure 7. Residual inactivated OT-I Tmem exhibit a PD-1^{hi}CD5^{hi} phenotype. A-C) OT-I Tmem were transferred to non-Tg mice that were irradiated (300cGy) and injected i.v. with BM from non-Tg or 11c.OVA donors (BMT) 1 week later. Controls (no BMT) were non-Tg or 11c.OVA mice injected with Tm in parallel with BM transfer test mice. 4 weeks after BM transfer, spleen cells were examined by flow cytometry. OT-I T cells were gated as CD45.1^{+}CD8^{+}Vα2^{+} lymphocyte-sized cells. Data are pooled from 2 experiments and show representative dot plots (**A**), histograms (**B**) or individual mice (**C**). Lines denote mean ± SEM (n=6).

Figure 1

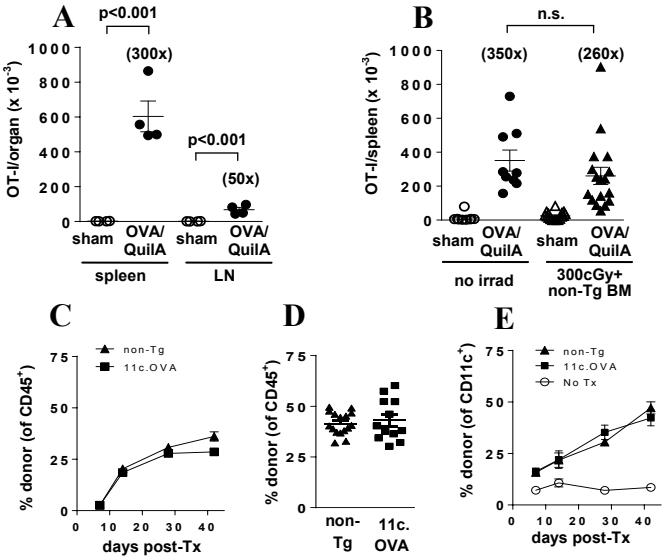

A
p<0.001
(300x)
p<0.001
(50x)
OT-I/organ (x 10^-3)
sham
OVA/ QuilA
sham
OVA/ QuilA
spleen
LN
B
n.s.
(350x)
(260x)
OT-I/spleen (x 10^-3)
sham
OVA/ QuilA
sham
OVA/ QuilA
no irrad
300cGy+ non-Tg BM
C
non-Tg
11c.OVA
% donor (of CD45+)
days post-Tx
D
% donor (of CD45+)
non-Tg
11c. OVA
E
non-Tg
11c.OVA
No Tx
% donor (of CD11c+)
days post-Tx

Figure 2

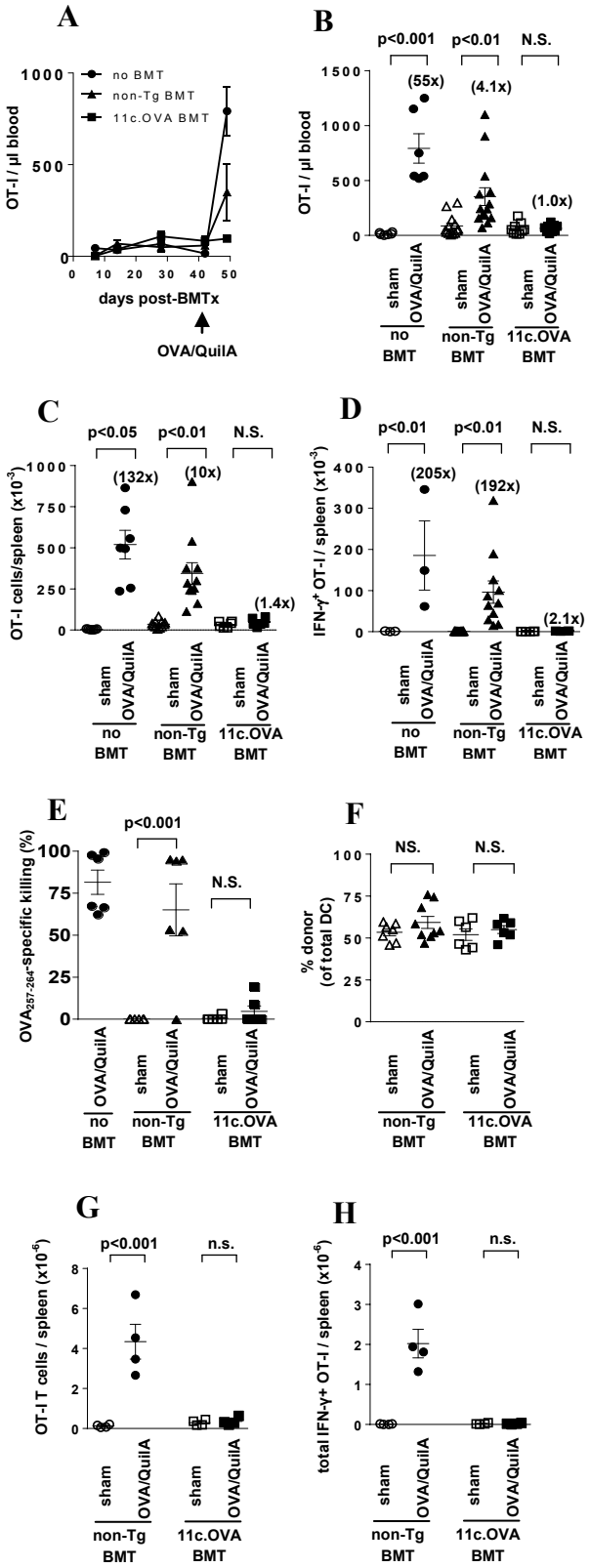

A
no BMT
non-Tg BMT
11c.OVA BMT
OT-I / µl blood
days post-BMTx
OVA/QuilA
B
p<0.001
p<0.01
N.S.
(55x)
(4.1x)
(1.0x)
OT-I / µl blood
sham
OVA/QuilA
no BMT
non-Tg BMT
11c.OVA BMT
C
p<0.05
p<0.01
N.S.
(132x)
(10x)
(1.4x)
OT-I cells/spleen (x10-3)
D
p<0.01
p<0.01
N.S.
(205x)
(192x)
(2.1x)
IFN-γ+ OT-I / spleen (x10-3)
E
p<0.001
N.S.
OVA257-264-specific killing (%)
F
NS.
N.S.
% donor (of total DC)
G
p<0.001
n.s.
OT-I T cells / spleen (x10-6)
H
p<0.001
n.s.
total IFN-γ+ OT-I / spleen (x10-6)

Figure 3

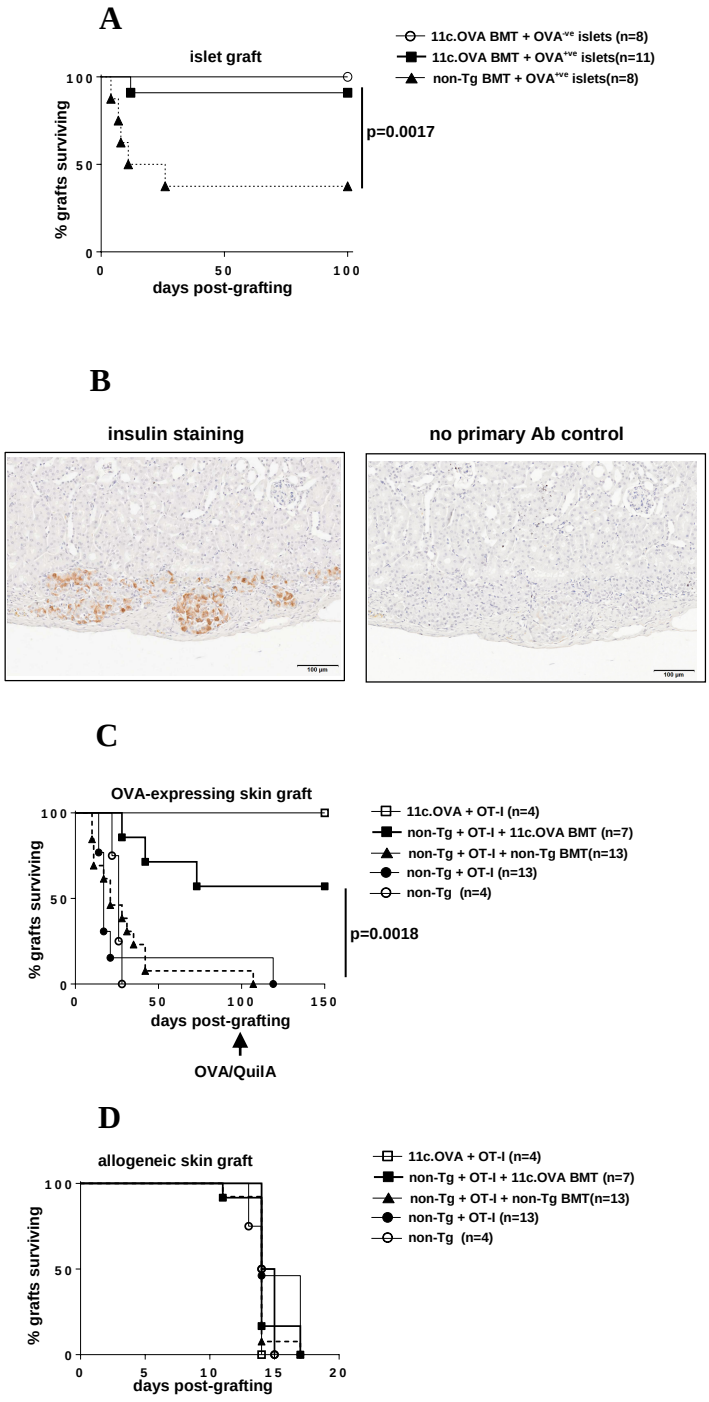

A
islet graft
11c.OVA BMT + OVA+ve islets (n=8)
11c.OVA BMT + OVA+ve islets(n=11)
non-Tg BMT + OVA+ve islets(n=8)
% grafts surviving
days post-grafting
p=0.0017
B
insulin staining
no primary Ab control
C
OVA-expressing skin graft
11c.OVA + OT-I (n=4)
non-Tg + OT-I + 11c.OVA BMT (n=7)
non-Tg + OT-I + non-Tg BMT(n=13)
non-Tg + OT-I (n=13)
non-Tg (n=4)
% grafts surviving
days post-grafting
p=0.0018
OVA/QuilA
D
allogeneic skin graft
11c.OVA + OT-I (n=4)
non-Tg + OT-I + 11c.OVA BMT (n=7)
non-Tg + OT-I + non-Tg BMT(n=13)
non-Tg + OT-I (n=13)
non-Tg (n=4)
% grafts surviving
days post-grafting

Figure 4

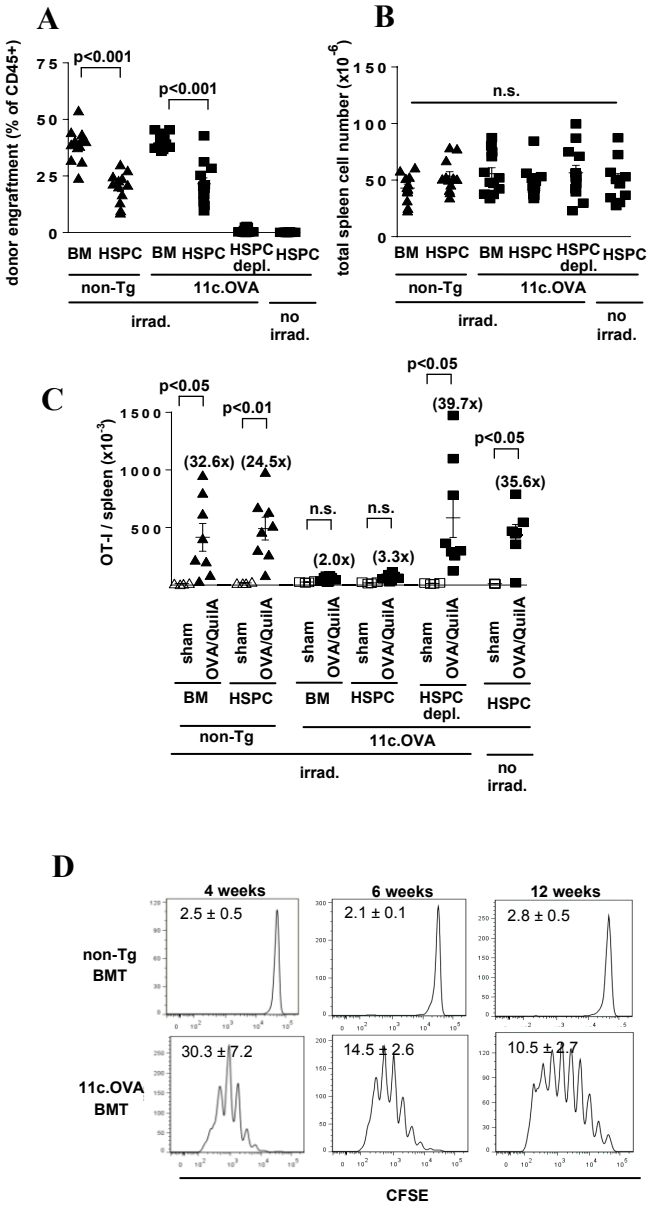

A
B
C
D
donor engraftment (% of CD45+)
p<0.001
p<0.001
BM HSPC BM HSPC HSPC depl. HSPC
non-Tg
11c.OVA
irrad.
no irrad.
total spleen cell number (x10^-6)
n.s.
OT-I / spleen (x10^-3)
p<0.05
p<0.01
(32.6x)
(24.5x)
n.s.
n.s.
(2.0x)
(3.3x)
p<0.05
(39.7x)
p<0.05
(35.6x)
sham
OVA/QuilA
BM
HSPC
HSPC depl.
4 weeks
6 weeks
12 weeks
non-Tg BMT
11c.OVA BMT
2.5 ± 0.5
2.1 ± 0.1
2.8 ± 0.5
30.3 ± 7.2
14.5 ± 2.6
10.5 ± 2.7
CFSE

Figure 5

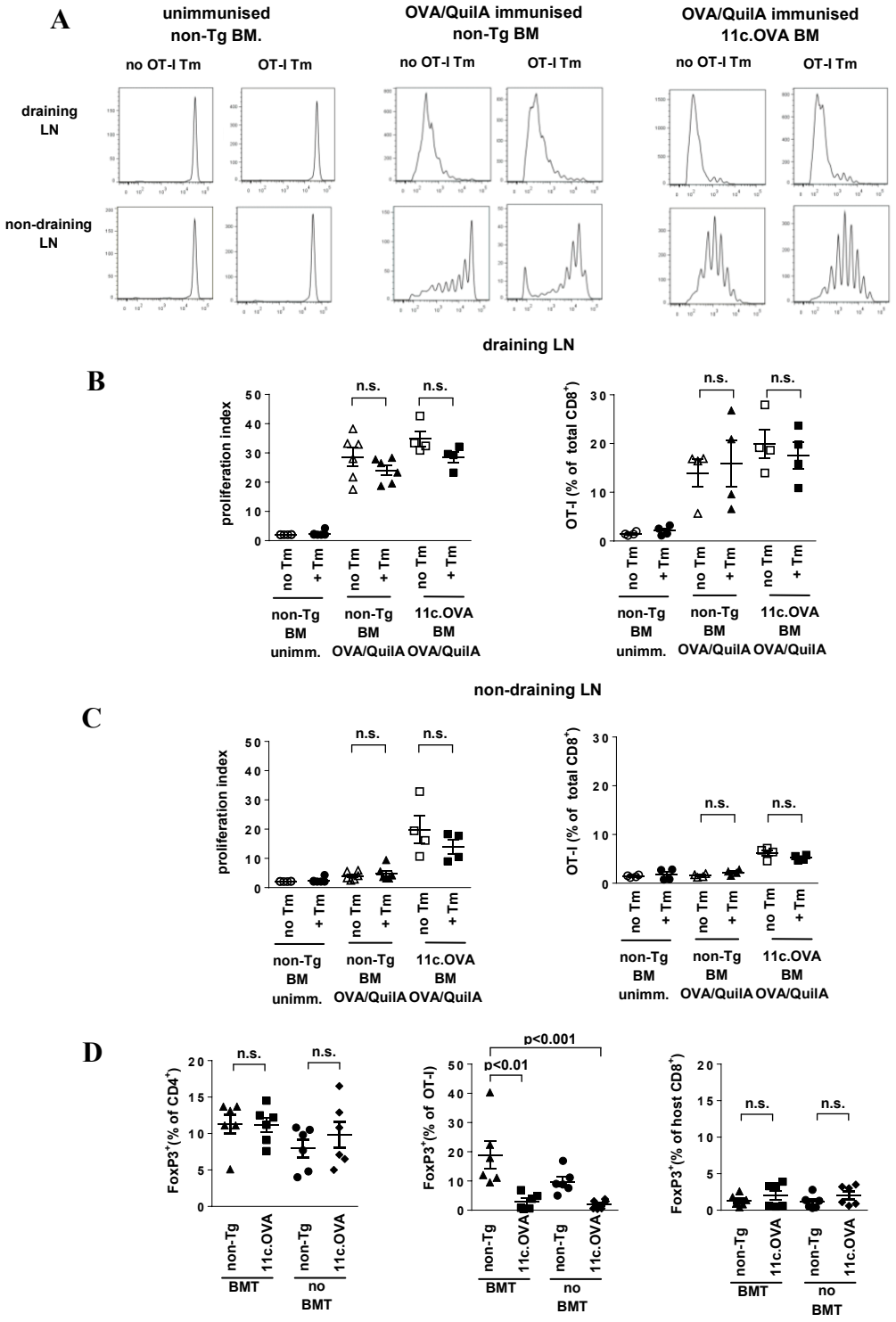

A
unimmunised non-Tg BM.
OVA/QuilA immunised non-Tg BM
OVA/QuilA immunised 11c.OVA BM
no OT-I Tm
OT-I Tm
draining LN
non-draining LN
B
draining LN
proliferation index
OT-I (% of total CD8+)
n.s.
no Tm
+ Tm
non-Tg BM unimm.
non-Tg BM OVA/QuilA
11c.OVA BM OVA/QuilA
C
non-draining LN
D
FoxP3+(% of CD4+)
FoxP3+(% of OT-I)
FoxP3+(% of host CD8+)
p<0.001
p<0.01
non-Tg
11c.OVA
BMT
no BMT

Figure 6

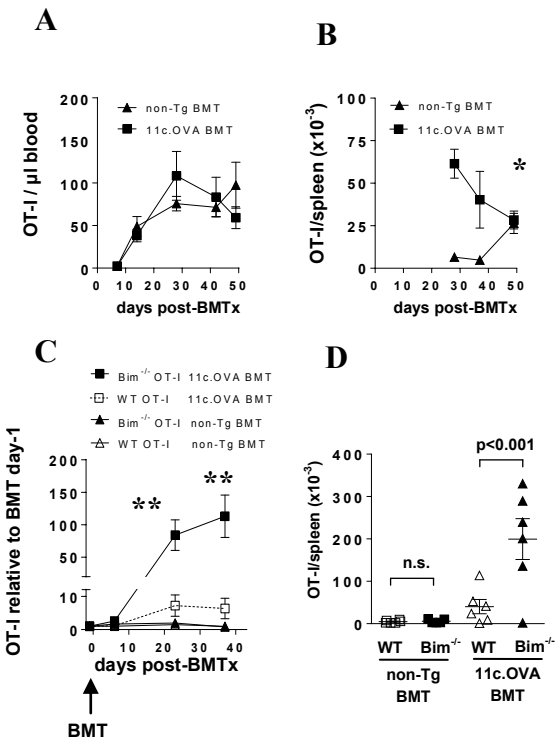
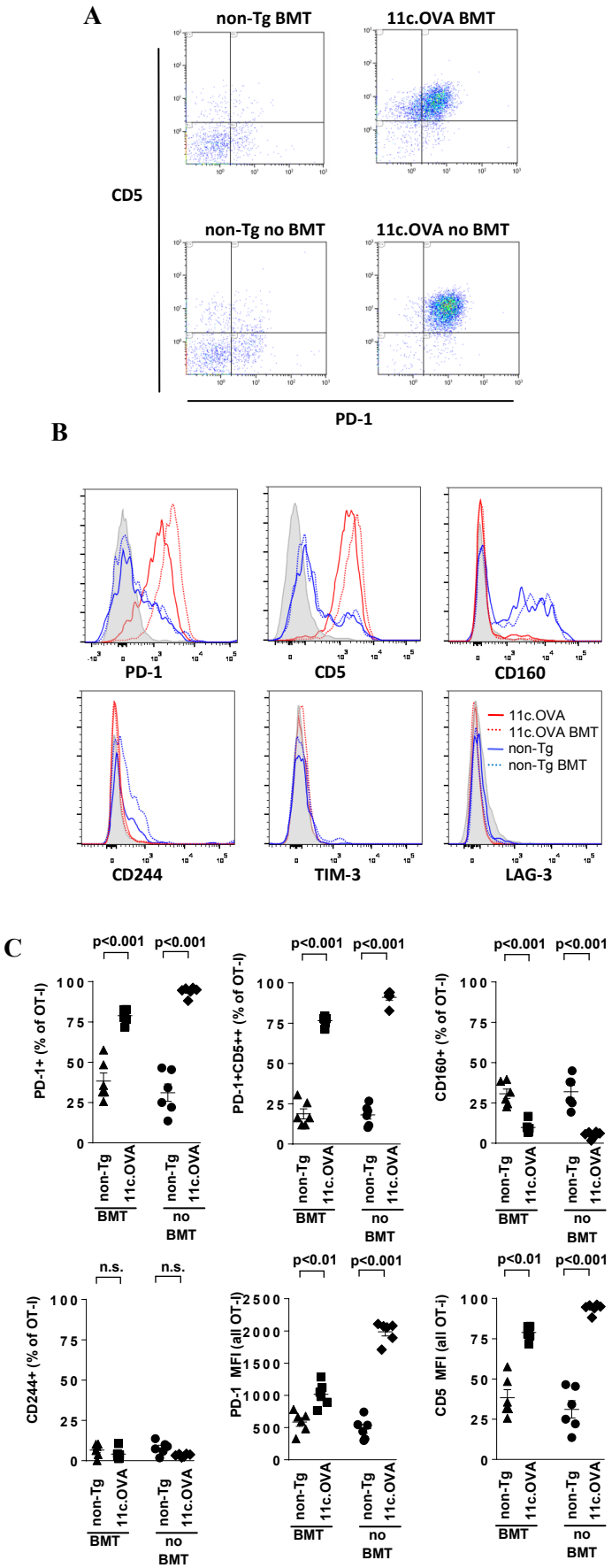
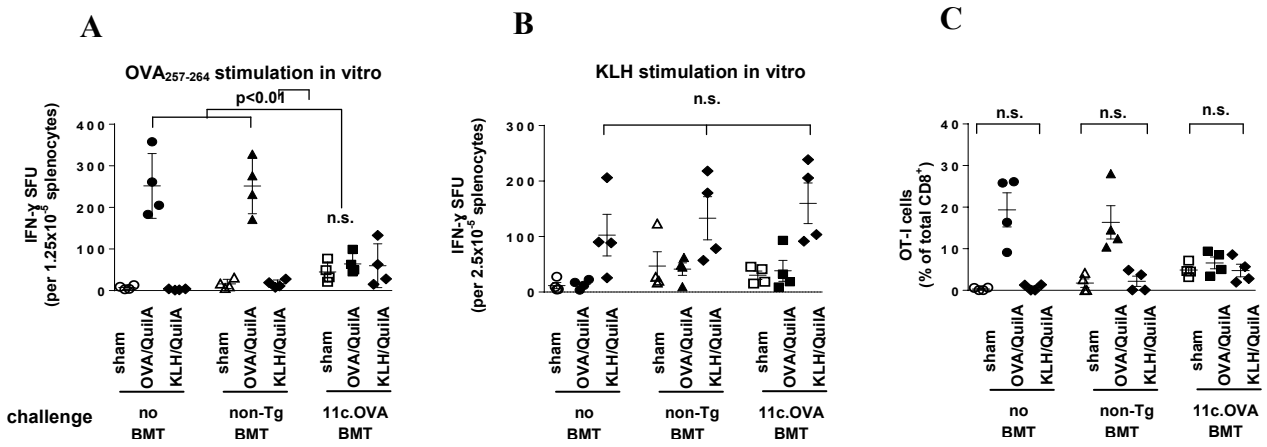

A
non-Tg BMT
11c.OVA BMT
OT-I / µl blood
days post-BMTx
B
non-Tg BMT
11c.OVA BMT
OT-I/spleen (x10-3)
*
days post-BMTx
C
Bim-/- OT-I 11c.OVA BMT
WT OT-I 11c.OVA BMT
Bim-/- OT-I non-Tg BMT
WT OT-I non-Tg BMT
OT-I relative to BMT day-1
**
**
days post-BMTx
BMT
D
OT-I/spleen (x10-3)
p<0.001
n.s.
WT
Bim-/-
WT
Bim-/-
non-Tg BMT
11c.OVA BMT

Figure 7

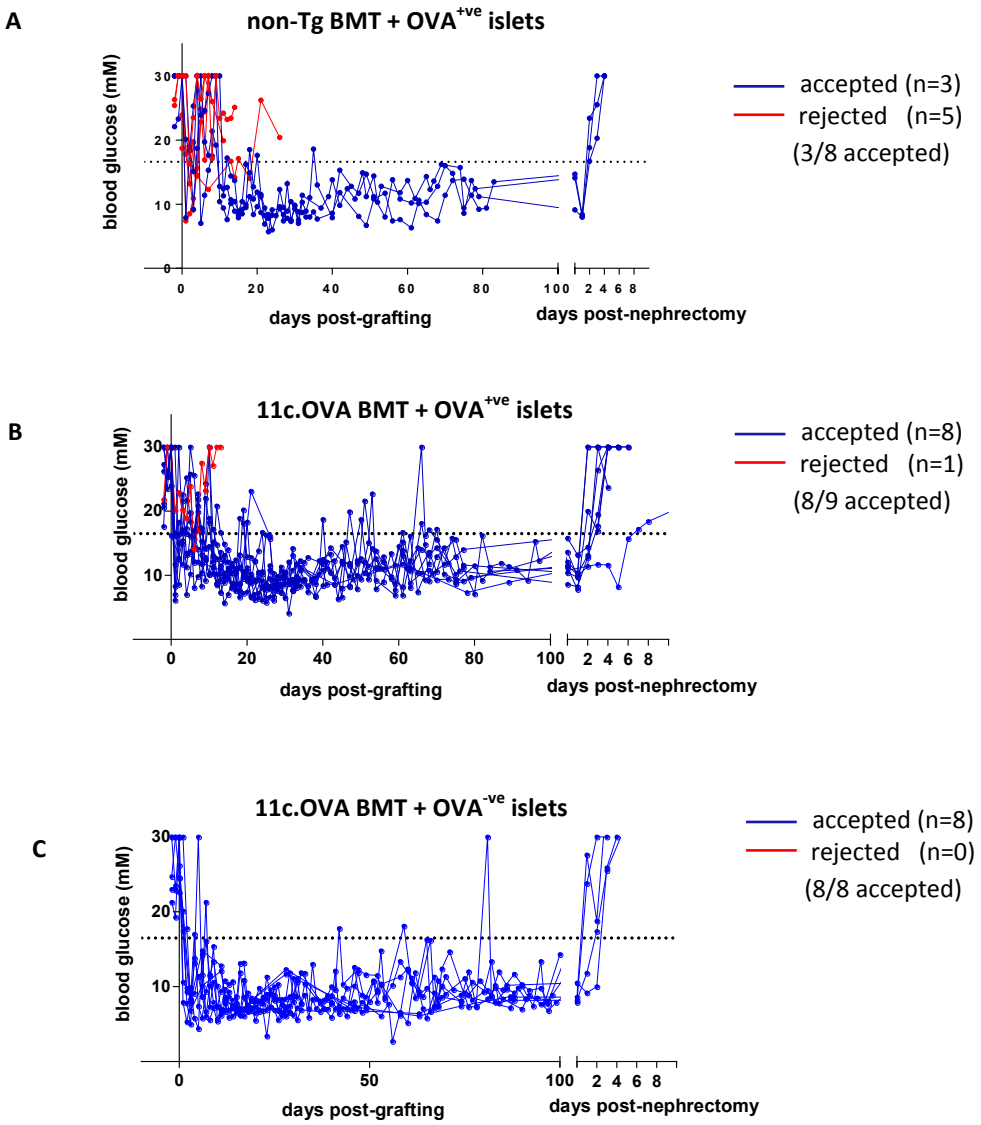

A
non-Tg BMT
11c.OVA BMT
non-Tg no BMT
11c.OVA no BMT
CD5
PD-1
B
PD-1
CD5
CD160
CD244
TIM-3
LAG-3
11c.OVA
11c.OVA BMT
non-Tg
non-Tg BMT
C
PD-1+ (% of OT-I)
PD-1+CD5++ (% of OT-I)
CD160+ (% of OT-I)
CD244+ (% of OT-I)
PD-1 MFI (all OT-I)
CD5 MFI (all OT-I)
p<0.001
p<0.01
n.s.
non-Tg
11c.OVA
BMT
no BMT



Supplementary Figure 1. Termination of CD8$^+$ Tmem responses is antigen-specific and establishes a long-lasting tolerogenic environment.

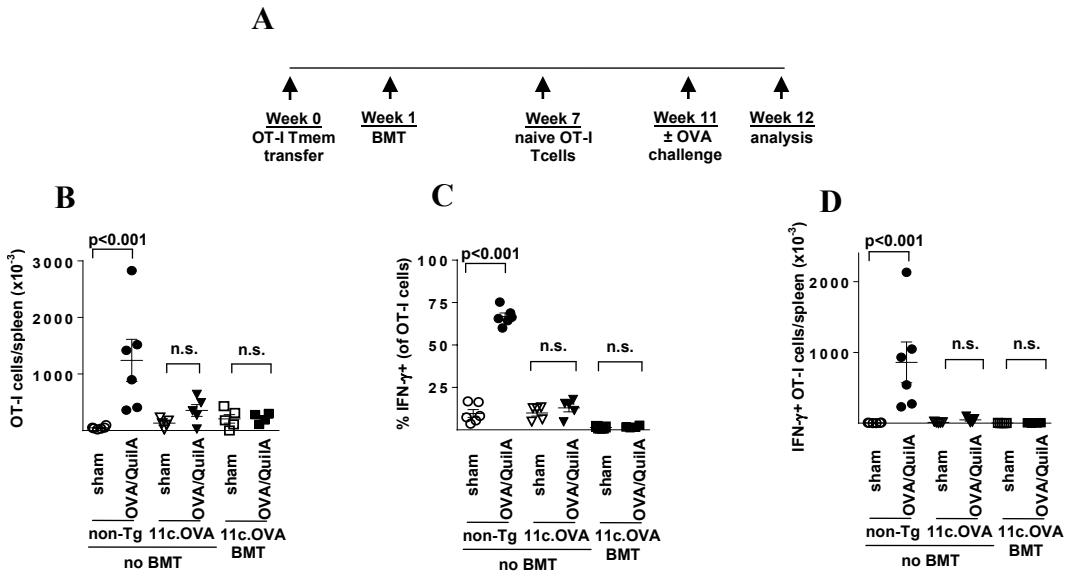
To determine if Tmem inactivation was antigen-specific we tested preservation of a bystander immune response. **A-C)** Non-Tg (CD45.1$^+$) mice were immunised with KLH/QuilA and 2 weeks later injected (i.v.) with OT-I Tmem (CD45.2$^+$). One week later mice were irradiated (300cGy) and non-Tg or 11c.OVA BM transferred. Unirradiated, non-transplanted mice (no BMT) were included as controls. Six weeks after BMT, mice were sham (PBS/QuilA), OVA (OVA/QuilA) or KLH (KLH/QuilA) challenged and a week later ELISpot performed using $OVA_{257-264}$ (**A**) or KLH (**B**) stimulation. OT-I T cells in spleen were enumerated flow cytometry (**C**). Data show individual mice (n=4 per group, mean ± SEM) pooled from 2 separate experiments of 2 per group.

In KLH-stimulated ELISpots, the frequency of IFN-γ spots did not differ significantly (**B**) indicating preservation of the immune response to KLH protein. KLH/QuilA challenge did not alter the frequency of OT-I T cells relative to sham-challenged controls (**C**).



Supplementary Figure 2. Stable glycemic control after islet transplant to OVA-encoding BM recipients.

A-D) OT-I Tmem ($4x10^7$) were transferred to non-transgenic C57BL/6 mice. One week later mice were irradiated (300cGy) and non-Tg (C57BL/6) or 11c.OVA BM transferred. Four weeks after BM transfer, mice were rendered diabetic by administration of streptozotocin (200mg/kg) and OVA^{+ve} islets from RIP.mOVA mice or OVA^{-ve} islets from littermate controls transplanted under the kidney capsule. Glycemia was monitored and rejection determined as 2 consecutive blood glucose readings >16.6mM. Mice carrying islet grafts were nephrectomised and blood glucose monitored post-nephrectomy as indicated. **A)** Test group in which mice received non-Tg (C57BL/6) BM transfer, **B)** Test group in which mice received OVA-encoding (11c.OVA) BM, **C)** Technical (no rejection) control group showing restoration of euglycemia by control OVA^{-ve} islets. Blue lines trace BG for 'accepted' grafts. Red lines trace BG for 'rejected' grafts.



Supplementary Figure 3. Transfer of antigen-encoding BM establishes a long-lasting tolerogenic environment.

To determine whether Ag-encoding BM transfer established a long-lasting tolerogenic environment BM recipients were probed with naive OVA-specific TCR transgenic OT-I T cells and tolerance induction tested.

A) Experimental outline. **B-D)** OT-I Tmem ($CD45.2^+$) were transferred to non-transgenic mice ($CD45.2^+$) and 1 week later mice were irradiated (300cGy) and injected i.v. with non-Tg or 11c.OVA BM. Six weeks after BMT, naive congenically-distinct (test) OT-I cells ($CD45.1^+$ $CD45.2^+$) were transferred to BMT recipients and additional control naive non-Tg and 11c.OVA mice. After a further 4 weeks, mice were sham- or OVA/QuilA-challenged. Test OT-I T cells ($CD8^+CD45.1^+CD45.2^+$) in spleen (**B**) and IFN-ɣ production (**C,D**) were determined by cytometry 1 week later. Data show individual mice (n=4 per group) pooled from 2 separate experiments of 2 per group Mean ± SEM (n=6) are shown.

Transferred (test) naive OT-I T cells are inactivated indicating the presence of a long-lasting tolerogenic environment in 11c.OVA BM recipients.